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(54) Title: METHOD OF PRODUCING HUMAN GROWTH FACTORS FROM WHOLE PLANTS OR PLANT CELL CULTURES

(57) Abstract

The production of hEGF is achieved in both whole plants and plant cell culture wherein the hEGF has a length of at least 200 amino acids. For epidermal growth factor this would comprise at least a tetramer of EGF units. Effectiveness or production of the translation process has been increased according to the present invention by (1) cloning of pre–pro–EGF cDNA of approximately 4.5 kb into both whole plants and cell culture to increase overall titers of active hEGF; (2) synthesizing cDNA and transforming plants and cell culture for production of an oligomeric polypeptide consisting of repeated hEGF domains; and (3) increasing the overall size of the gene to be expressed with a fusion construct encoding hEGF linked to a protein that is efficiently produced in plant systems. As needed, synthetic cDNA includes plant–specific proteolytic cleavage sites between EGF repeats to facilitate correct processing *in planta*. Appropriate proteolytic cleavage sites upstream and downstream of hEGF are added if needed to obtain final product. In whole plants, use of a regulatory element confers hEGF production characteristics into traditionally non–saleable portions of crop plants, such as the leafy tops of potatoes. Use of potato tops under post–harvest conditions, results in overexpression production of hEGF in non–saleable plant portions towards the end of the harvesting season, without affecting crop quality.

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METHOD OF PRODUCING HUMAN GROWTH FACTORS FROM WHOLE PLANTS OR PLANT CELL CULTURES

FIELD OF THE INVENTION

The present invention relates generally to a method for producing human growth factors from whole plants or plant cell culture. More specifically, the invention relates to producing a human growth factor from a plant cell encoded to produce the human growth factor with a length of at least 200 amino acids from transgenic plant cells.

BACKGROUND OF THE INVENTION

Growth factors and monoclonal antibodies (Mabs) are diverse yet highly specialized types of proteins having research and commercial applications in areas of therapeutics and diagnostics.

Therapeutic uses of human epidermal growth factor (hEGF) include treatment of soft tissue wounds (U.S. 5,218,093, 1993), specifically including skin and eye injuries as well as corneal and stomach ulcers (Frost and Sullivan 1996, 1994). In addition, several hEGF-bearing fusion constructs have been considered and/or tested, including mitotoxins for treatment of restenosis (Frost and Sullivan, 1994) and radioconjugates for a variety of anti-neoplastic therapies (Grieg et al., 1988).

Current production techniques for these proteins such as hybridoma and other types of mammalian cell culture methods (Köhler and Milsten, 1975) are generally slow, labor intensive, and consequently, expensive. In addition, current production techniques are difficult to validate due to the pathogenic and oncogenic potential of cultivated mammalian tissue.

Multimers of from 2 to 7 EGF units each having 53 amino acid residues have been produced from bacterial hosts, eg E. coli, Streptomyces and Bacillus, fungal hosts, eg Saccharomyces, Pichia and Aspergillus, insect cell host, and

mammalian cell hosts, eg CHO cells and COS cells. (U.S. Patent No. 5218093, 1993). hEGF production in *Staphylococcus aureus* (U.S. Patent No. 5004686, 1991) is by a fusion construct encoding hEGF linked to a protein. Synthesis methods using transgenic bacterial strains have problems such as faulty antibody gene expression, protein folding difficulties, inability to glycosylate proteins, and relegation of foreign peptides to insoluble material accumulated in inclusion bodies.

Transgenic plants can be used for the production of high value, medicinally important proteins, for example, production of Mabs (Hiatt et al., 1989; Düring et al., 1990; Benvenuto et al. 1991, Firek et al. 1993, Gao et al. 1993), human growth hormone (Kay et al. 1986) and human serum albumin (Sijmons et al. 1990). Transformed cells synthesize, secrete, and accumulate functional antibodies including single (Benvenuto et al. 1991) and double (Düring et al. 1990, Hiatt et al. 1991) domain immunoglobulins. However, it is noted that none of these authors investigated production of any human growth factor from transgenic plants.

Plant cell culture media are well-defined and inexpensive compared to mammalian cell culture media. Further, plant cell products, unlike mammalian-derived protein formulations, are generally assumed as neither pathogenic nor oncogenic to humans (Crawford, 1995). Also, when compared to similar production in transgenic bacterial strains (Attaai and Shuler 1987), plant tissue culture methods showed greater stability of foreign gene expression, even without use of selection pressure (Gao et al. 1991). One author, Higo et al. (1993) produced a human growth factor, specifically hEGF in transgenic tobacco with cDNA fragment size of 180 bp. Unsatisfactory foreign peptide levels of 20 to 60 pg/mg (ppb) total soluble leaf protein were obtained. This is despite the fact that plant progeny appeared to produce high levels of hEGF mRNA. Exact reasons for low observed levels of hEGF production are unclear. However, no signal peptide was encoded upstream of hEGF cDNA which could cause the foreign protein to be

relegated to the cytosol. Within this cell fraction, hEGF suffers proteolytic attack, especially considering the relatively small size (53 amino acids) or the peptide.

Although advantages have been observed for deriving proteins including EGF from plants, no transgenic plant cell culture process has been commercially developed for production of human growth factor. The lack of commercial exploitation of plant derived proteins is due in part to existing technological hurdles as observed by Higo et al. In addition, Ma et al., 1995 reported Mab titers of up to 500 μg/g (ppm) fresh weight of plant material (or 300 mg/L on a cell culture basis) whereas comparable mammalian cell processes are reported to attain levels of 1-2 g/L and higher (Rosenberg, personal communication, 1995). Implementation of alternative production systems to mammalian and bacterial culture, such as plant cellular techniques, has been further limited by non-technological factors, such as industry and regulatory acceptance (Simonsen and McGrogan, 1994) because of the investment made in developing and validating the more established non-plant methods.

Accordingly, there is a continuing need for plant based production of human growth factors.

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SUMMARY OF THE INVENTION

Despite the hurdles in technology development and commercialization, economic analysis indicates that regulatory costs associated with plant cell culture may reduce by as much as $70,000 per batch as compared to analogous mammalian cell processes (Crawford, 1995). In addition, direct production costs for whole plant processes at equal protein production rates appear to be two to four orders-of-magnitude lower than comparable mammalian cell processes (Agracetus 1995). Additionally, as plant cell titers increase, this type of production becomes even more capital cost-effective.

It is, therefore, an object of the present invention to provide whole plant and plant cell culture derived human growth factors at higher overall concentrations and production rates, comparable to mammalian host cell systems.

It is a further object of the present invention to synthesize specific human growth factors.

It is another object of the present invention to increase production rates and concentrations by increasing protein stability through the use of fusion constructs.

It is a further object of the present invention to use *Nicotiana tabacum* (tobacco) and *Solanum tuberosum* (potato) whole plants and highly synchronous suspensions.

According to the present invention, the production of human growth factors is achieved in whole plants or plant cell culture wherein the human growth factor is produced with a length of at least 200 amino acids. For epidermal growth factor this would comprise at least a tetramer of EGF units.

Modifying chimeric cDNA and subcloning into a plant expression vector are done using standard molecular cloning procedures (Ausubel et al. 1992) and splicing PCR techniques (Marks et al. 1992).

Effectiveness or production of the translation process has been increased according to the present invention by (1) cloning of pre-pro-EGF cDNA of approximately 4.5 kb into both whole plants and cell culture to increase overall titers of active hEGF, (2) synthesizing cDNA and transforming plants and cell culture for production of an oligomeric polypeptide consisting of repeated hEGF domains, and (3) increasing the overall size of the gene to be expressed with a fusion construct encoding hEGF linked to a protein that is efficiently produced in plant systems. As needed, synthetic cDNA includes plant-specific proteolytic cleavage sites between EGF repeats to facilitate correct processing *in planta*. Appropriate proteolytic cleavage sites upstream and downstream of hEGF are added if needed to obtain final product.

The subject matter of the present invention is particularly pointed out and distinctly claimed in the concluding portion of this specification. However, both the organization and method of operation, together with further advantages and objects thereof, may best be understood by reference to the following description taken in connection with accompanying drawings wherein like reference characters refer to like elements.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides the size of EGF precursor (pre-pro-EGF) relative to correctly processed EGF.

FIG. 2 depicts schematically the construction of pZD203, a vector used to modify the restriction sites on pre-pro-EGF to develop cDNA suitable for cloning into the plant expression vector pGA643.

FIG. 3 depicts schematically the construction of pZD204, the plant expression vector carrying pre-pro-EGF.

FIG. 4 shows EGF levels seen in individual calli resulting from positive transformation and antibiotic selection. EGF concentrations were determined using enzyme-linked immunosorbent assay and are based on a 30 KD protein size.

DESCRIPTION OF THE PREFERRED EMBODIMENT(s)

The present invention is a method for production of human growth factors using whole plants as well as plant cell suspensions transformed with appropriately constructed vector plasmids, wherein the human growth factor is produced with a length of at least 200 amino acids. More specifically, the method of the present invention is stable expression of human growth factors of interest as direct therapeutics, targeted delivery systems and research reagents. Human growth factors produced include human epidermal growth factor (hEGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor (TNF), heparin-binding epidermal growth factor (HBEGF), insulin-like growth factor (ILGF), platelet-derived endothelial cell growth factor (PDECGF), platelet-derived angiogenesis factor (PDAF), and bone-and-cartilage inducing growth factor (BCIF).

Any plant from the plant kingdom may be utilized. Specific types of plants that are amenable to the transformation steps listed herein include, but are not limited to monocotyledonous, dicotyledonous, and tuberous plants. Preferred species include but are not limited to *Nicotiana tabacum* (tobacco), *Solanum tuberosum* (potato), *Glycine max* (soybean), and *Zea mays* (corn).

The method of the present invention, a method of producing human growth factors from plant cells, has the steps of:

(a) obtaining a positive transformant of the plant cells, the positive transformant carrying genetic material encoding the production of a human growth factor with a length of at least 200 amino acids;

(b) cultivating the positive transformant; and

(c) obtaining the human growth factors.

The step of obtaining may be as simple as purchasing or more complex actual making by well known methods, for example direct particle bombardment as described in Gene Transfer by Particle Bombardment, Klein TM, Knowlton S, Arentzen R, Plant Tissue Culture Manual, D1, pp 1-12, 1991, Kluwer Academic Publishers, or by Agrobaterium mediated transformation as described in Hoekema et al. 1985 (Hoekema KM, Hirsch PR, Hooykaaf PJJ, Schliperoort RA, 1985, Nononcogenic Plant Vectors for Use in the Agrobacterium Binary System, Plant Molecular Biology, Vol. 5, 85-89), and further described herein.

The step of cultivating involves either whole plant cultivating or tissue cultivating by any of well known cultivating methods.

The step of obtaining is by well known separation purification steps, for example ultrafiltration, affinity chromatography, and/or electrophoresis.

An Agrobacterium mediated transformation method of the present invention has the steps of:

(a) modifying chimeric cDNA encoding a specific growth factor for subcloning into a plant expression vector

(b) subcloning the chimeric cDNA into the plant expression vector;

(c) transferring the plant expression vector containing transgenic plant cells to an agrobacterium;

(d) co-cultivating a portion of the transgenic plant cells (suspension culture or leaf disks) with the agrobacterium;

(e) selecting positive transformants from the co-cultivated culture on an antibiotic selective media;

(f) permitting growth of the transgenic plant cells in whole plants or suspensions; and

(g) extracting a liquid containing the human growth factor;

wherein the improvement comprises:

said human growth factor having a length of at least 200 amino acids.

Modifying chimeric cDNA and subcloning into a plant expression vector are done using standard molecular cloning procedures (Ausubel et al. 1992) and splicing PCR techniques (Marks et al. 1992). More specifically, modifying chimeric cDNA, has the steps of:

(a) adding a transcription promoter to the upstream or 5' end of the chimeric cDNA; and

(b) adding a transcription terminator to the downstream or 3' end of the chimeric cDNA. The transcription promoter and the transcription terminator are regulatory elements.

Further, an additional regulatory element encoding a signal peptide may be added between the transcription promoter and the 5' end of the chimeric cDNA in order to relegate the product human growth factor to a specific cellular organelle. In addition, other regulatory elements may be added either between the promoter and the additional regulatory element encoding the signal peptide or at the 3' end of the chimeric cDNA to obtain greater mRNA stability between transcription and translation events.

In either whole plants or cell cultures, to enhance expression of the chimeric gene (hEGF), the present invention further includes manipulation of a 35S promoter by duplication of the upstream region (-343 to -90 bp) of the CaMV 35S promoter to increase transcription activity, as well as use of TSC29 and TSC40 promoters. These promoters and their transcription activity have been reported by Gao et al. 1994, and Dai et al. 1995.

In whole plants, transcription promoters may include the upstream enhancer (nucleotides -343 to -90 relative to the transcription start site) of the CaMV 35S promoter (Benfey et al. 1989) or the chlorophyll a/b binding protein (*cab1*) promoter (Ha and An 1988). Use of these types of regulatory elements confers human growth factor production characteristics into traditionally non-salable portions of crop plants, such as the leafy tops of potatoes. Use of potato tops, for example, under post-harvest conditions, results in overexpression and production

of human growth factor in non-salable plant portions towards the end of the harvesting season, without affecting crop quality.

Transferring the plant expression vector into the agrobacterium is completed using the freeze-thaw method (An 1987). For monocotyledonous species, super-binary vectors, such as pTOK233 and pSB131, are used to achieve high transformation frequency (Ishida et al. 1996). Remaining cocultivation, selection, growth, and extraction steps (d through g) have been described by Magnusen et al. (1996), and are well known in the art of plant molecular biology.

Many human growth factors possess relatively short lengths of between 50 and 100 amino acids. For example, hEGF has a length of 53 amino acids. Accordingly, obtaining a larger construct of at least 200 amino acids requires either (1) cloning the larger precursor cDNA, (2) synthesizing a concatemer consisting of multiple gene copies encoding the growth factor, or (3) increasing the overall size of a gene to be expressed using a fusion construct encoding a growth factor linked to a protein that is efficiently produced in plant systems.

An example of obtaining a larger precursor to increase the overall protein size is the cDNA encoding pre-pro-EGF. This particular gene, at approximately 4.5 kb, encodes a 1207 amino acid protein that, *in vivo*, is proteolytically cleaved to yield 53 amino acid EGF. In plant systems, this larger protein will provide additional stability against proteolytic degradation.

Synthesizing the cDNA concatemer is preferably done by ligating multiple gene copies using peptide linkers to obtain a processed protein length of at least 200 amino acids. The multiple gene copies are preferably an oligomeric polypeptide having of repeated growth factor cDNA domains. Peptide linkers may be used that are (1) proteolytically cleaved *in planta*, (2) proteolytically cleaved in a separate enzymatic treatment step, or (3) resistant to proteolytic cleavage. Peptide linkers that are proteolytically cleaved by serine proteases *in planta* preferably possess the amino acid sequence Arg-Asn. This sequence already exists when EGF is concatemerized since the C-terminal amino acid is arginine and the N-terminal amino acid is asparagine. To achieve *in planta* cleavage, the processed

protein is targeted either to the cell cytosol (no signal peptide) or vacuole (phytohemagglutinin signal peptide [Chrispeels et al.1991]). To achieve proteolytic cleavage in a separate enzymatic treatment step, the same amino acid sequence is preferably used (Arg-Asn) and the growth factor concatemer is either targeted to the chloroplast (pea photosystem II signal peptide) or secreted (PR-II signal peptide) to limit proteolytic degradation. To achieve resistance to proteolytic cleavage, linkers would preferably possess the amino acid sequence Arg-Pro. This sequence is resistant to serine proteases. Specifically for EGF, linkage would preferably be achieved by synthesizing cDNA encoding a single proline unit between growth factor monomers cDNA.

Increasing the overall size of a gene may be done by ligating EGF with cDNA encoding a protective protein to protect from proteolytic cleavage, thereby forming a fusion construct. Protective proteins include but are not limited to streptococcal protein G or -galactosidase, that have both been shown to inhibit proteolysis when attached to the C-terminus of other foreign proteins (Hellebust et al. 1989). Gene size could also be increased by ligating EGF with cDNA encoding another protective protein of commercial interest that processes well in plant-based systems. Protective proteins further include human serum albumin (Sijmons et al. 1990) and phytase (Verwoerd et al. 1995).

At least one genetic regulatory element may be included in the cDNA encoding the transcription of specific growth factors. Regulatory elements include transcription promoters or enhancers that increase the frequency of transcription events, leader sequences that increase the stability of mRNA prior to translation, and signal peptides that target proteins to specific organelles for posttranslational modifications and accumulation. Examples of transcription enhancers include but are not limited to the octapine synthase enhancer, a 16 bp palindrome (ACGTAAGCGCTTACGT) (Ellis et al. 1987) and the B-domain of the cauliflower mosaic virus 35S promoter (Kay et al. 1987). An example of a leader sequence includes but is not limited to alfalfa mosaic virus RNA4 leader sequence (Jobling and Gehrke 1987). Examples of signal peptides include but are not

limited to the tobacco PR-S signal peptide (Cornelissen et al. 1986) and the phytohemagglutinin signal peptide (Hunt and Chrispeels 1991).

Example 1

The bacteriophage λEGF116 (ATCC No. 59956) containing the gene encoding the full length polypeptide of human kidney pre-pro-EGF was obtained from ATCC. Pro-EGF (**FIG. 1**) is the 1207 amino acid precursor in which hEGF is flanked by polypeptide segments of 907 and 184 residues at its NH_2- and COOH-termini, respectively (Bell et al., 1986). The remainder of the 4.8 kb pre-pro-EGF gene encodes native signal peptides at both the NH_2- and COOH- termini of pro-EGF. The polypeptide contains a transmembrane (TM) binding region that facilitates proper cleavage in the endoplasmic reticulum.

The full length of cDNA was excised with Sma I, Hind III, and Eco RI restriction enzymes, as shown on **FIG. 2**, producing two separate fragments. These were sequentially ligated into compatible Sma I and Eco RI sites in pBluescript- creating the 7.5 kb plasmid pZD203. After proper orientation was confirmed, pre-pro-EGF cDNA was further excised with Xba I and Cla I restriction enzymes and ligated into compatible sites located between the CaMV 35S promoter and T_7 transcription terminator of binary vector pGA643, forming the 16 kb plasmid pZD204 (**FIG. 3**). This plasmid was directly transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (An 1987). The transferred plasmid was introduced into tobacco whole plants (by leaf disks) and calli (by suspension culture) by co-cultivation with the Agrobacterium thereby producing transformants. Over 200 specific samples of transformants were taken from the co-cultivation and separately placed on kanamycin selective media. The co-cultivated transformants that grew were positive transformants. The positive transformants were screened under kanamycin selection pressure and preliminary ELISA results indicated the presence of hEGF in tobacco calli. Accumulation levels of hEGF in select transgenic calli are shown on a ng/g fresh weight basis in **FIG. 4**. The bars in FIG. 4 represent a random sample of the specific samples of

transformants. The highest level of accumulation at approximately 400 ng/(g fresh weight cells) (ppb) corresponds to a concentration of 4.1 ng/(mg total soluble protein) (ppm) (based on a measured total soluble protein level of approximately 98 mg/(g fresh weight cells)). The 4.1 ng/(mg total soluble protein) (ppm) corresponds to 4100 pg/(mg total soluble protein) (ppb) which is almost two orders-of-magnitude greater than the result of 60 pg/(mg total soluble protein) (ppb) reported by Higo et al. (1993).

Further ELISA and Northern blot analyses were used to detect high levels of foreign protein production and mRNA transcription, respectively. Western blot analysis, completed to determine protein size, showed that specific EGF bearing constructs of 30 KD were produced. This size corresponds to approximately 250 amino acids.

Closure

While a preferred embodiment of the present invention has been shown and described, it will be apparent to those skilled in the art that many changes and modifications may be made without departing from the invention in its broader aspects. The appended claims are therefore intended to cover all such changes and modifications as fall within the true spirit and scope of the invention.

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(1) GENERAL INFORMATION:
(i) APPLICANT: Brian S. Hooker, et al
(ii) TITLE OF INVENTION: Method of Producing Human Growth Factors From Whole Plants or Plant Cell Cultures
(iii) NUMBER OF SEQUENCES: 8
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Paul W. Zimmerman
(B) STREET: P.O. Box 999
(C) CITY: Richland
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 99352
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3 1/2 Magnetic Disk
(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: WORD97
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/747,246
(B) FILING DATE: 11-12-96
(C) CLASSIFICATION: unknown
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: N/A
(B) FILING DATE:
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Paul W. Zimmerman
(B) REGISTRATION NUMBER: 34,761
(C) REFERENCE/DOCKET NUMBER: E-1519
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 509-375-2981
(B) TELEFAX: 509-375-2592
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4481bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: double strands
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
(A) DESCRIPTION: Sense orientation of complementary DNA for pro-EGF
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE: 5'-AGT GAC TCA GTC GAG ... TTC TCA CTC GTC-3 end
(v) FRAGMENT TYPE: 4.5kb SmaI/HindIII double strands DNA fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: kidney
(B) STRAIN: human
(C) INDIVIDUAL ISOLATE: GI Belle
(D) DEVELOPMENTAL STAGE: adult
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: fetal human liver library
(B) CLONE: lambda CH4A; lambda EMBL4; lambda GM1416
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
(ix) FEATURE:
(A) NAME/KEY: human epithelial growth factor cDNA
(B) LOCATION:
(C) IDENTIFICATION METHOD: cross-hybridization with mouse cDNA
(D) OTHER INFORMATION:
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
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FROM ____(position) TO ____ (position)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCT GGT GTC TCA GTG ATC ATG GAT TTT CAT TAT AAT GAG AAA AGA ATC TAT TGG 332
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GTA GCA GTT GAT CCA GTA GAA AGG TTT ATA TTT TGG TCT TCA GAG GTG GCT GGA 602
AGC CTT TAT AGA GCA GAT CTC GAT GGT GTG GGA GTG AAG GCT CTG TTG GAG ACA 656
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TTT GTA CCA CTT GGT GAA CTG AAA GTA GTG CAT CCA CTT GCA CAA CCC AAG GCA 980
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CTC CAA GGC AGC ATG CTG AAG CCC TCA TCA CTG GTT GTG GTT CAT CCA TTG GCA 2276
AAA CCA GGA GCA GAT CCC TGC TTA TAT CAA AAC GGA GGC TGT GAA CAT ATT TGC 2330
AAA AAG AGG CTT GGA ACT GCT TGG TGT TCG TGT CGT GAA GGT TTT ATG AAA GCC 2384
TCA GAT GGG AAA ACG TGT CTG GCT CTG GAT GGT CAT CAG CTG TTG GCA GGT GGT 2438
GAA GTT GAT CTA AAG AAC CAA GTA ACA CCA TTG GAC ATC TTG TCC AAG ACT AGA 2492
GTG TCA GAA GAT AAC ATT ACA GAA TCT CAA CAC ATG CTA GTG GCT GAA ATC ATG 2546
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CCT GCC TCC TCC AAG TGC ATC AAC ACC GAA GGT GGT TAT GTC TGC CGG TGC TCA 2762
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GCA TTG GAC AAG TAT GCA TGC AAC TGT GTT GTT GGC TAC ATC GGG GAG CGA TGT 3032
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CCC CAG TTA TGT GGA ATG GGC ACA GAG CAA GGC TGC TGG ATT CCA GTA TCC AGT 3518
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CAG TGA                                                              3686

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(3) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 783bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: double strands
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
(A) DESCRIPTION: sense orientation of five copies of mature EGF concatemers
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE: 5'-CGC GTC AAG GGT ... TCT CAG TGA TAA-3 end
(v) FRAGMENT TYPE: 4.5kb SmaI/HindIII double strands DNA fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: kidney
(B) STRAIN: human
(C) INDIVIDUAL ISOLATE: Z.Dai, et al.
(D) DEVELOPMENTAL STAGE: adult
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: fetal human liver library
(B) CLONE: lambda CH4A;lambdaEMBL4; lambda GM1416
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
(ix) FEATURE:
(A) NAME/KEY: Concatemer of mature EGF fragment without linker
(B) LOCATION:
(C) IDENTIFICATION METHOD: PCR cloning
(D) OTHER INFORMATION:
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:
FROM ____(position) TO ____ (position)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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ATC GGG GAG CGA TGT CAG TAC CGA GAC CTG AAG TGG TGG GAA CTG CGC AAT AGT 324
GAC TCT GAA TGT CCC CTG TCC CAC GAT GGG TAC TGC CTC CAT GAT GGT GTG TGC 378
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GGG GAG CGA TGT CAG TAC CGA GAC CTG AAG TGG TGG GAA CTG CGC AAT AGT GAC 486
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GAG CGA TGT CAG TAC CGA GAC CTG AAG TGG TGG GAA CTG CGC AAT AGT GAC TCT 648
GAA TGT CCC CTG TCC CAC GAT GGG TAC TGC CTC CAT GAT GGT GTG TGC ATG TAT 702
ATT GAA GCA TTG GAC AAG TAT GCA TGC AAC TGT GTT GTT GGC TAC ATC GGG GAG 756
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(4) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 891bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: double strands
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
(A) DESCRIPTION: sense orientation concatemer of mature EGF fragments with linkers
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE: 5'-GTC CAG AGC ... CAG TGA TAA-3 end
(v) FRAGMENT TYPE: 5-copies of 159bp concatemer mature EGF linked with linkers
(vi) ORIGINAL SOURCE:
(A) ORGANISM: kidney
(B) STRAIN: human
(C) INDIVIDUAL ISOLATE: Z. Dai, et al
(D) DEVELOPMENTAL STAGE: adult
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
(ix) FEATURE:
(A) NAME/KEY: concatemer of mature EGF linked with linkers
(B) LOCATION:
(C) IDENTIFICATION METHOD:PCR cloning
(D) OTHER INFORMATION: Cleavage sites at 142-165, 307-331, 465-489, 631-655.
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
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(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:
FROM ____(position) TO ____ (position)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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GCA TGC AAC TGT GTT GTT GGC TAC ATC GGG GAG CGA TGT CAG TAC CGA GAC CTG 324
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GAA TGT CCC CTG TCC CAC GAT GGG TAC TGC CTC CAT GAT GGT GTG TGC ATG TAT 432
ATT GAA GCA TTG GAC AAG TAT GCA TGC AAC TGT GTT GTT GGC TAC ATC GGG GAG 486
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CAT GAT GGT GTG TGC ATG TAT ATT GAA GCA TTG GAC AAG TAT GCA TGC AAC TGT 648
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(5) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 330bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double strands
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
(A) DESCRIPTION: upstream enhancer (from -343 to -90 bp) of 35S promoter
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FRAGMENT TYPE: 253bp upstream of 35S promoter enhancer element
(vi) ORIGINAL SOURCE:
(A) ORGANISM: cauliflower mosaic virus (CaMV)
(B) STRAIN: Cabb B-D
(C) INDIVIDUAL ISOLATE: Z.Dai, et al
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY:
(B) CLONE:
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
(ix) FEATURE:
(A) NAME/KEY: 35S promoter B-domain enhancer
(B) LOCATION:
(C) IDENTIFICATION METHOD: standard cloning
(D) OTHER INFORMATION: B-domain of 35S promoter from EcoR V site to Hind II site (upstream enhancer region from -343 to -90 bp)
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:
FROM ____(position) TO ____ (position)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(6) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1441bp
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: double strands
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
(A) DESCRIPTION: 5'-untranscription region of chl a/b binding protein
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FRAGMENT TYPE: 11kb EcoR 1 fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: whole plants
(B) STRAIN: Arabidopsis
(C) INDIVIDUAL ISOLATE: Ha et al
(D) DEVELOPMENTAL STAGE: 30 day old seedlings
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: genomic DNA library
(B) CLONE: lambda bAT1005
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
(ix) FEATURE:
(A) NAME/KEY: arabidopsis cab1 gene promoter
(B) LOCATION:
(C) IDENTIFICATION METHOD: cross-hybridization
(D) OTHER INFORMATION:
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
(F) PAGES:
(G) DATE:
(H) DOCUMENT NUMBER:
(I) FILING DATE:
(J) PUBLICATION DATE:
(K) RELEVANT RESIDUES IN SEQ ID NO:
FROM ____(position) TO ____ (position)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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AAGCACAATT TTTCTTTTTT ACAATCACTA GACCTCACAG GCTCACACAT ATGCTTTAGA 180
GCACATTCTA AACTTTGAAC TATAAAAGCT GTTAACACTA ATACACTATG CGTTCTTTTT 240
TGCTCCAAAC ACTTTTGATC CATTATTAGG AGACACTCCA CTTAGAAAGA TTTTCTAATC 300
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TGACCCTGAC CATCACCTTG ATCTGGTGGA TTCCAATGTT TTCTTCTTCT TCTAATAAAA 780
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T 1441

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(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FRAGMENT TYPE: Alu 1 (from 7143bp)-EcoRl(to 7517bp)
(vi) ORIGINAL SOURCE:
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(B) STRAIN: cM4-184
(C) INDIVIDUAL ISOLATE: RJ Shepherd
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
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(H) CELL LINE:
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(B) MAP POSITION:
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(ix) FEATURE:
(A) NAME/KEY: CaMV 35S promoter
(B) LOCATION:
(C) IDENTIFICATION METHOD: cross-hybridization
(D) OTHER INFORMATION:
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
(E) ISSUE:
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(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FRAGMENT TYPE: 1500bp BamH-Hind lll
(vi) ORIGINAL SOURCE:
(A) ORGANISM: tobacco NT1 cells
(B) STRAIN:
(C) INDIVIDUAL ISOLATE: Z.Dai, et al
(D) DEVELOPMENTAL STAGE: 3 days old
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE: NT1
(I) ORGANELLE:

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(B) CLONE: TSC 40
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(C) IDENTIFICATION METHOD: plaque hybridization
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(C) JOURNAL:
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(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE:
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(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FRAGMENT TYPE: 253bp HindIII/EcoRV fragment + 343bp HindIII/EcoRI fragment
(vi) ORIGINAL SOURCE:
(A) ORGANISM: whole cell
(B) STRAIN: CM4-184
(C) INDIVIDUAL ISOLATE: Z.Dai, et al
(D) DEVELOPMENTAL STAGE:
(E) HAPLOTYPE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:
(I) ORGANELLE:
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(B) CLONE: POS-1
(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT:
(B) MAP POSITION:
(C) UNITS:
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(A) NAME/KEY: CaMV 35S promoter with duplication of upstream B domain
(B) LOCATION:
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:
(x) PUBLICATION INFORMATION:
(A) AUTHORS:
(B) TITLE:
(C) JOURNAL:
(D) VOLUME:
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CTCTAGAGGA TCCCCGGGTG GTCAGT                                     1159
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CLAIMS

We claim:

1. A method of producing human growth factors from plant cells, comprising the steps of:

(a) obtaining a positive transformant of the plant cells, the positive transformant carrying genetic material encoding the production of a human growth factor with a length of at least 200 amino acids;

(b) cultivating the positive transformant; and

(c) obtaining the human growth factors.

2. The method as recited in claim 1, wherein obtaining the positive transformant has the step of:

modifying a chimeric cDNA encoding the human growth factor with a length of at least 200 amino acids, for subcloning into a plant expression vector.

3. The method as recited in claim 2, further comprising the steps of:

(a) subcloning the chimeric cDNA into the plant expression vector and obtaining a subcloned plant expression vector;

(b) transferring the subcloned plant expression vector into a plurality of plant cells;

(c) selecting a plurality of positive transformants from the plurality of plant cells on an antibiotic selective media;

(d) permitting growth of the portion of the plurality of plant cells in whole plants or suspensions; and

(e) extracting a liquid containing the human growth factor from the plurality of transgenic plant cells.

4. The method as recited in claim 3, wherein transferring is by direct particle bombardment.

5. The method as recited in claim 3, wherein transferring is by Agrobacterium mediated transformation.

6. The method as recited in claim 5, wherein Agrobacterium mediated transformation comprises the steps of:

(a) placing the subcloned plant expression vector to an agrobacterium;

(b) co-cultivating the Agrobacterium containing the subcloned plant expression vector with the plurality of plant cells.

7. The method as recited in claim 1, wherein the step of cultivating is with a whole plant.

8. The method as recited in claim 1, wherein the step of cultivating is with a plant tissue culture.

9. The method as recited in claim 1, wherein the step of obtaining is selected from the group consisting of ultrafiltration, affinity chromatography, and electrophoresis.

10. The method as recited in claim 1, wherein the length of at least 200 amino acids is obtained by cloning a cDNA.

11. The method as recited in claim 10, wherein said cDNA is a pre-pro-EGF cDNA.

12. The method as recited in claim 1, wherein the length of at least 200 amino acids is obtained by synthesizing a cDNA.

13. The method as recited in claim 12, wherein said synthesizing is concatomerizing multiple gene copies to obtain the length of at least 200 amino acids.

14. The method as recited in claim 1, further comprising increasing an overall size of a gene to be expressed with a fusion construct encoding an hEGF linked to a protein that is efficiently produced in plant systems.

15. The method as recited in claim 1, wherein said human growth factor is selected from the group consisting of epidermal growth factor (EGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor (TNF), heparin-binding epidermal growth factor (HBEGF), insulin-like growth factor (ILGF), platelet-derived endothelial cell growth factor (PDECGF), platelet-derived angiogenesis factor (PDAF), and bone-and-cartilage inducing growth factor (BCIF) and combinations thereof.

16. The method as recited in claim 2, wherein modifying is by adding a regulatory element selected from the group consisting of leader sequences, signal peptides, transcription promoters or enhancers, and transcription terminators.

17. The method as recited in claim 2, wherein modifying a chimeric cDNA, comprises the steps of:

(a) adding said transcription promoter to the upstream or 5' end of the chimeric cDNA; and

(b) adding said transcription terminator to the downstream or 3' end of the chimeric cDNA.

18. The method as recited in claim 17, further comprising adding an additional regulatory element encoding a signal peptide, said additional regulatory

element added between the transcription promoter and the upstream 5' end of the chimeric cDNA.

19. The method as recited in claim 18, further comprising adding a regulatory element between the transcription promoter and the additional regulatory element encoding the signal peptide to enhance mRNA stability.

20. The method as recited in claim 18, further comprising adding a regulatory element at the downstream or 3' end of the chimeric cDNA to enhance mRNA stability.

21. The method as recited in claim 17, wherein transcription promoters limit growth factors production to a non-crop portion of a transgenic whole plant.

22. The method as recited in claim 21, wherein the transcription promoters are selected from the group consisting of an upstream enhancer region (-343 to -90 bp) of a CaMV 35S promoter, a chlorophyll a/b binding promoter (cab1) and combinations thereof.

23. The method as recited in claim 17, wherein the transcription promoters are selected from the group consisting of a modified 35S promoter, TSC29 promoter, TSC40 promoter and combinations thereof.

24. The method as recited in claim 23, wherein the modified 35S promoter is a 35S promoter modified by duplicating an upstream enhancer region (-343 to -90 bp) of the 35S promoter to increase transcription activity.

25. The method as recited in claim 2, wherein said cDNA is a pre-pro-EGF cDNA.

26. The method as recited in claim 25, wherein said pre-pro-EGF cDNA has approximately 4.5 kb, whereby overall titers of active hEGF in both whole plants and cell culture are increased.

27. The method as recited in claim 2, wherein the length of at least 200 amino acids is obtained by synthesizing the cDNA.

28. The method as recited in claim 27, wherein said synthesizing is concatomerizing multiple gene copies to obtain the length of at least 200 amino acids.

29. The method as recited in claim 28, wherein said multiple gene copies are an oligomeric polypeptide having of repeated hEGF domains.

30. The method as recited in claim 2, further comprising increasing an overall size of a gene to be expressed with a fusion construct encoding an hEGF linked to a protein that is efficiently produced in plant systems.

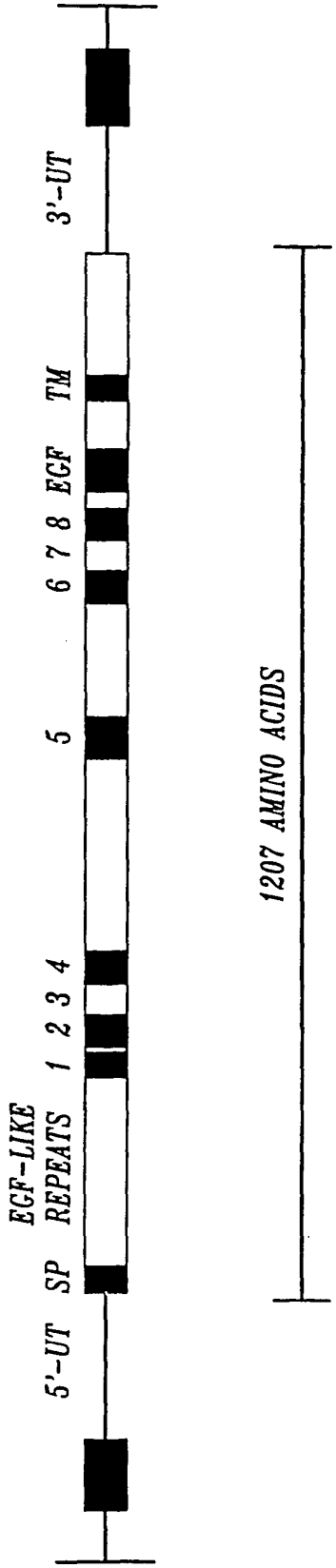
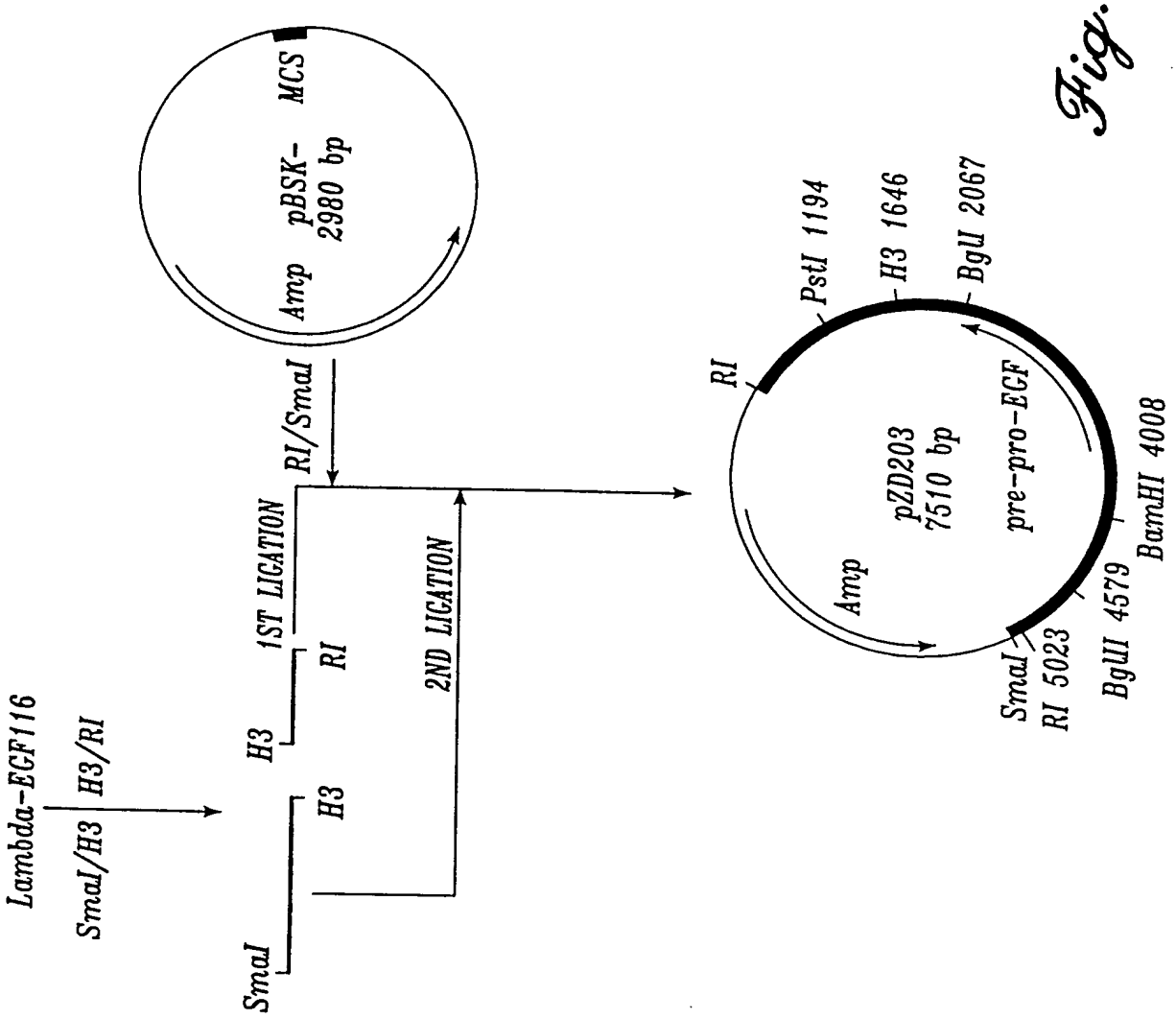


Fig. 1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RI
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Fig. 2

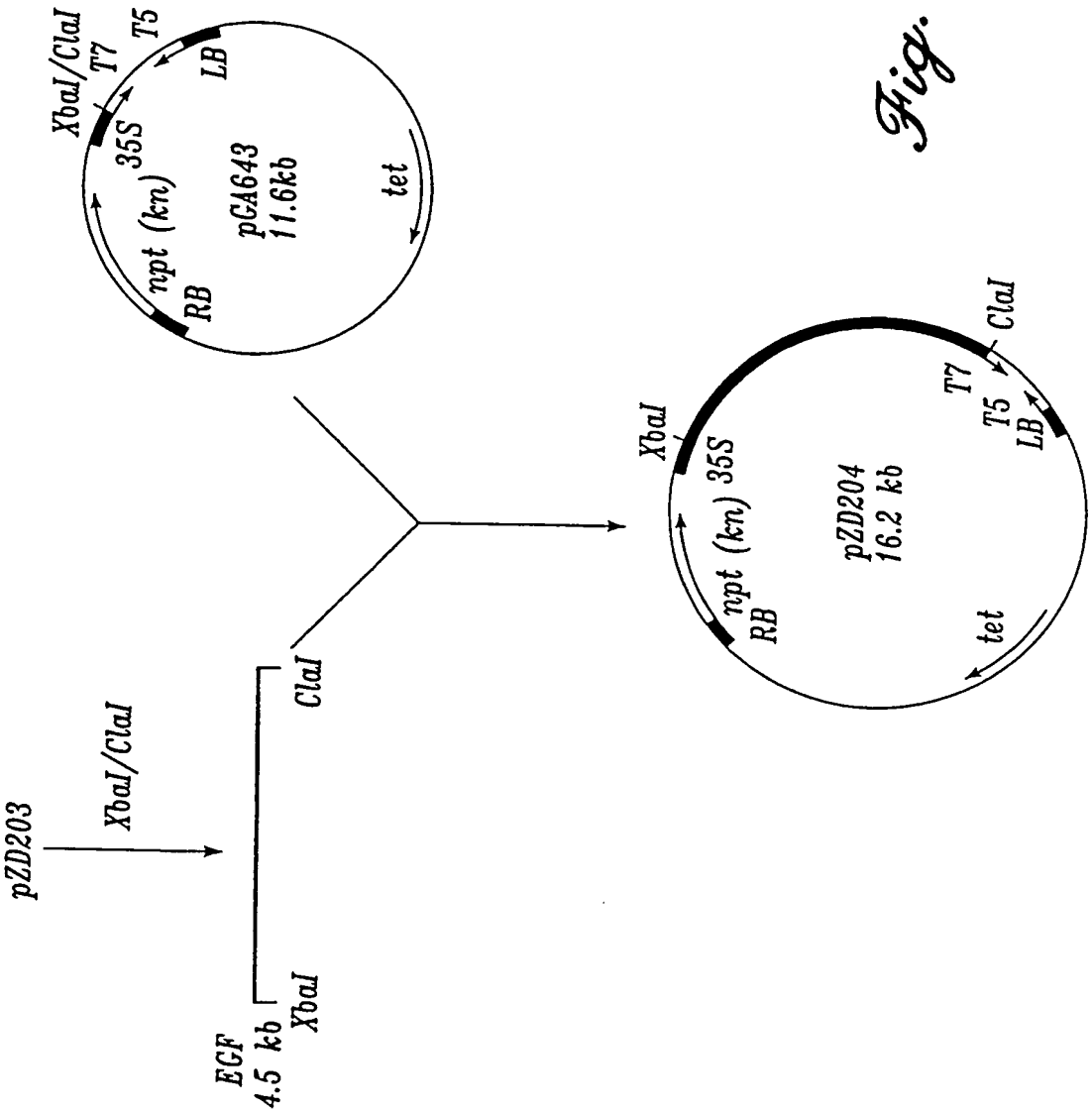
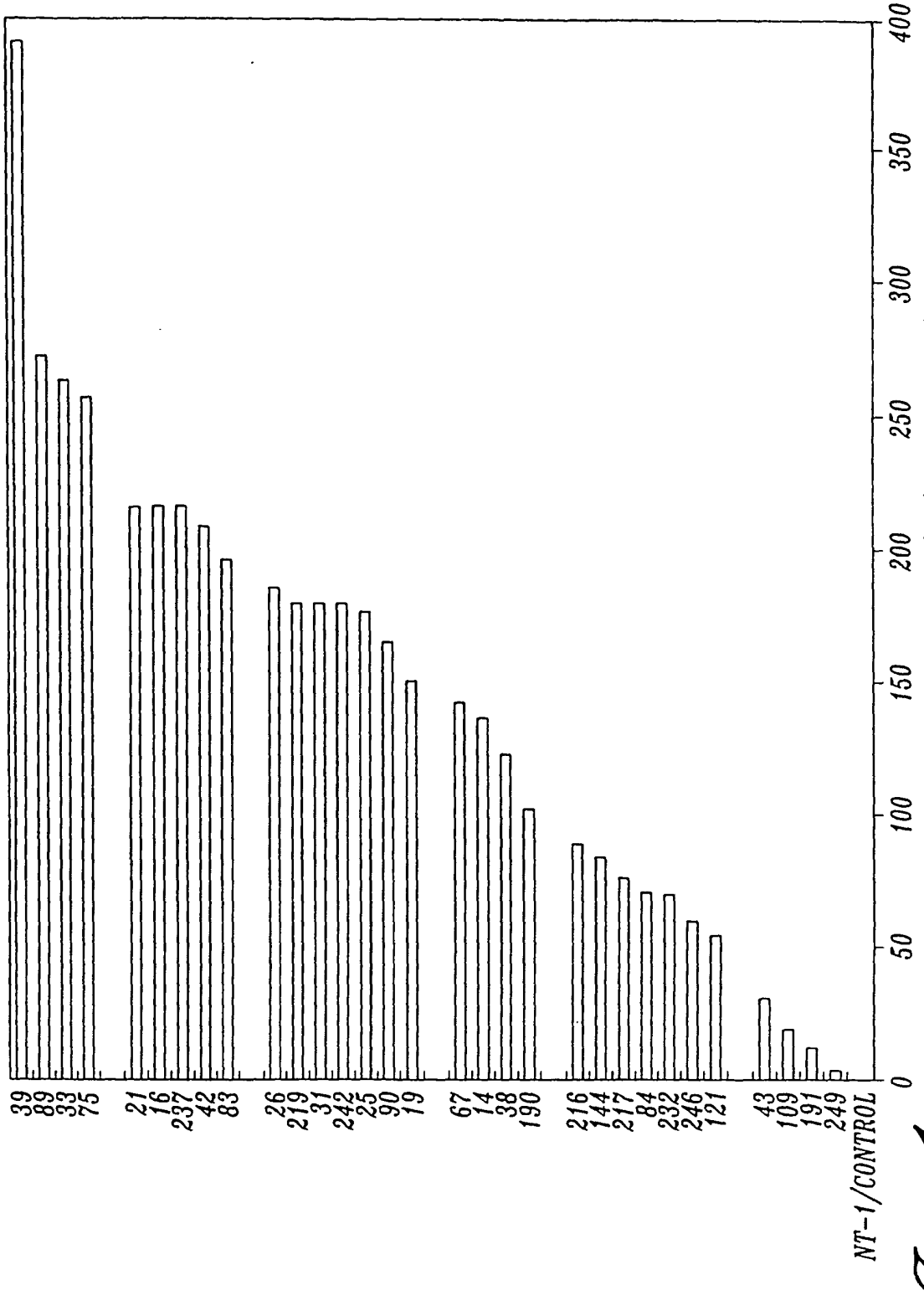


Fig. 3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200
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300
350
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26 219 31 242 25 90 19
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43 109 191 249

Fig. 4

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ɔnal Application No
PCT/US 97/20603

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE 3687705 A	18-03-93
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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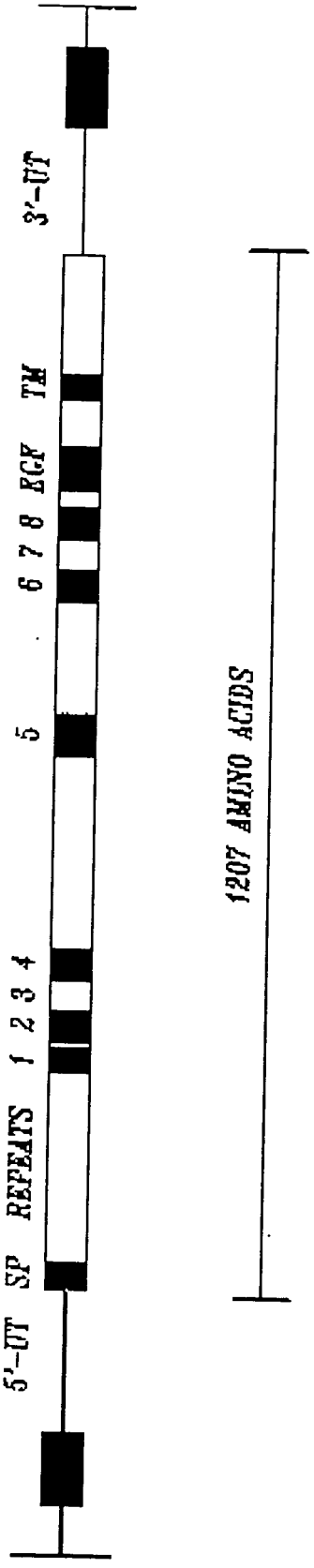

Fig. 1

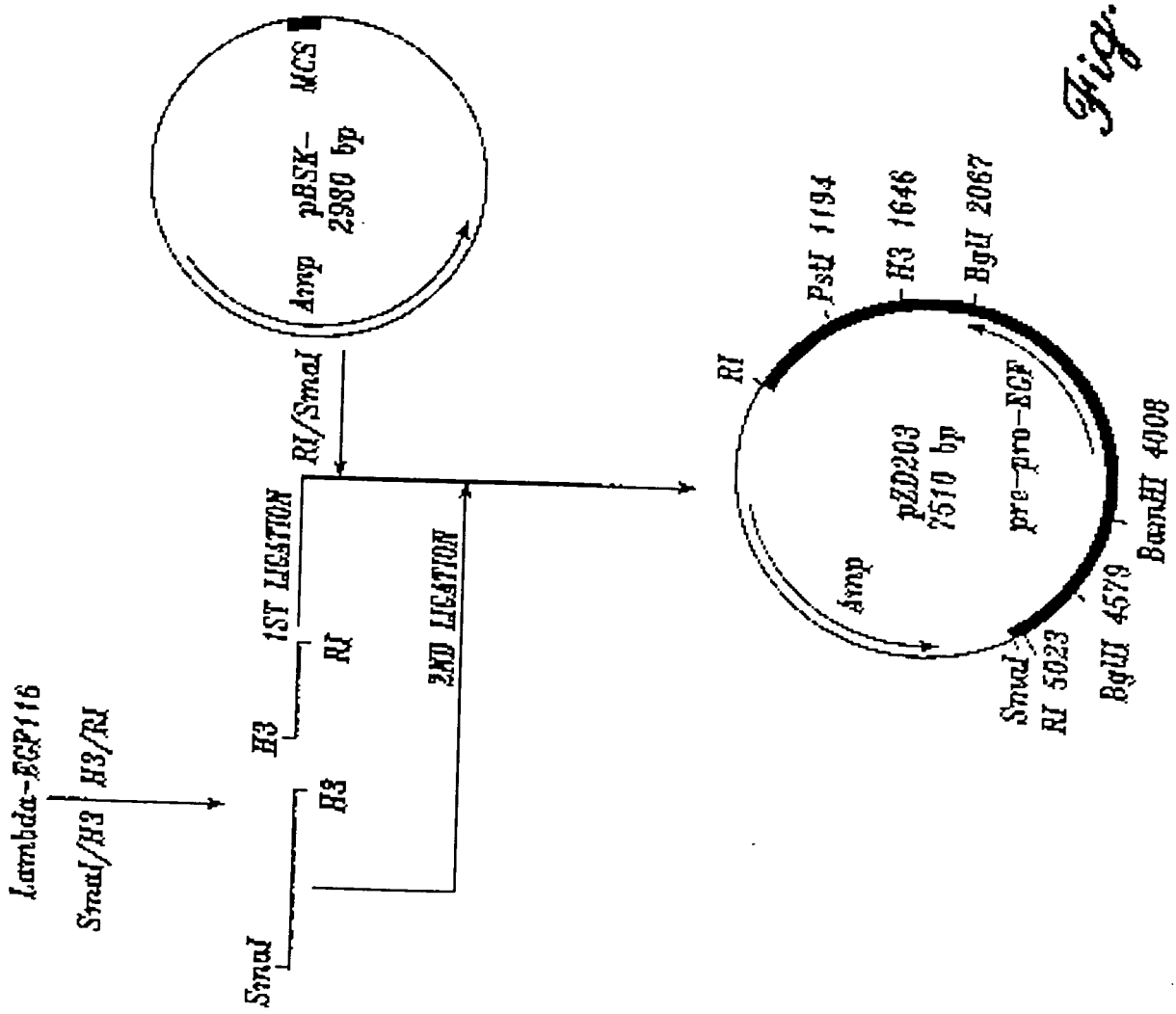
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Fig. 2

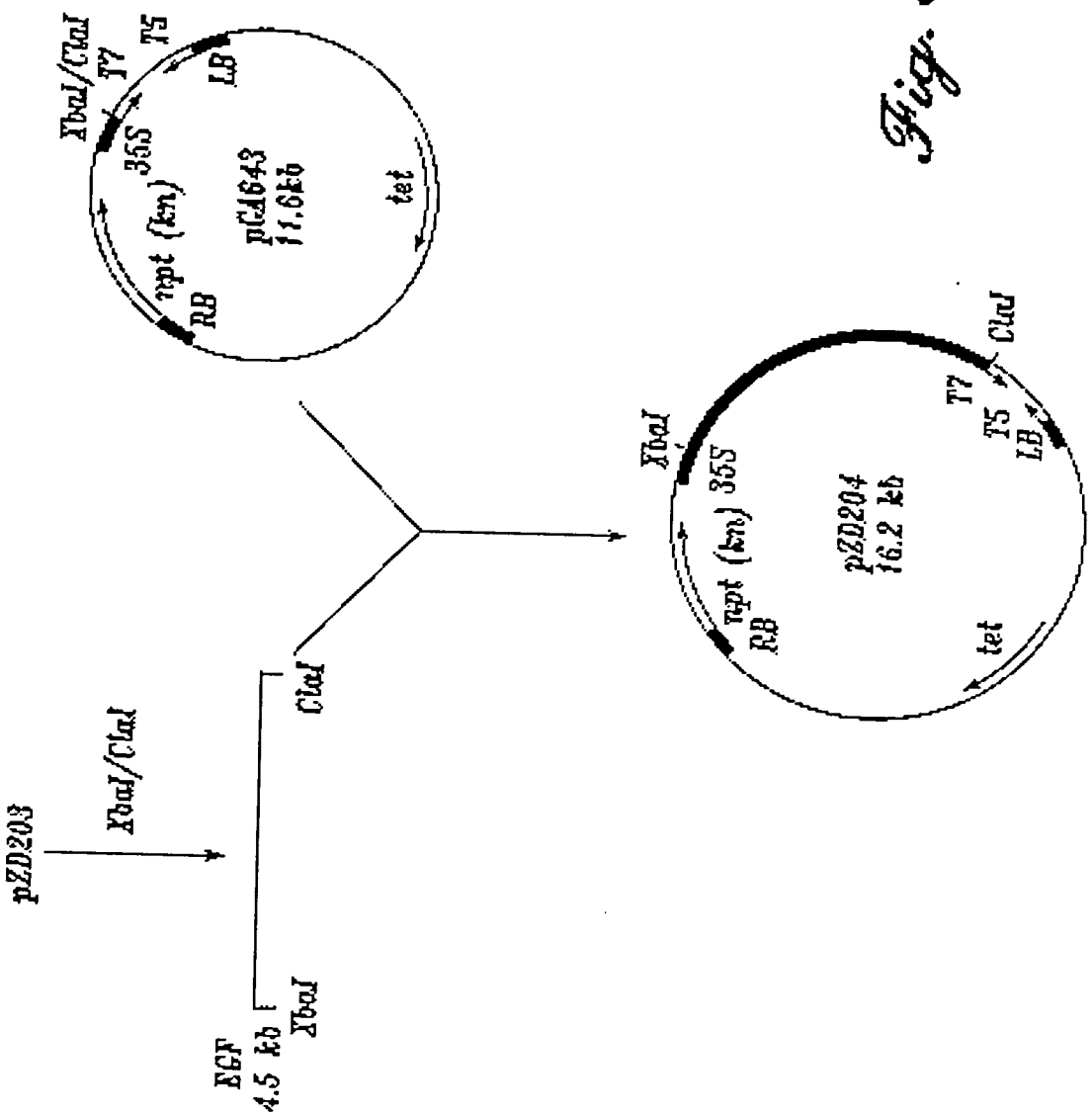
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Fig. 3

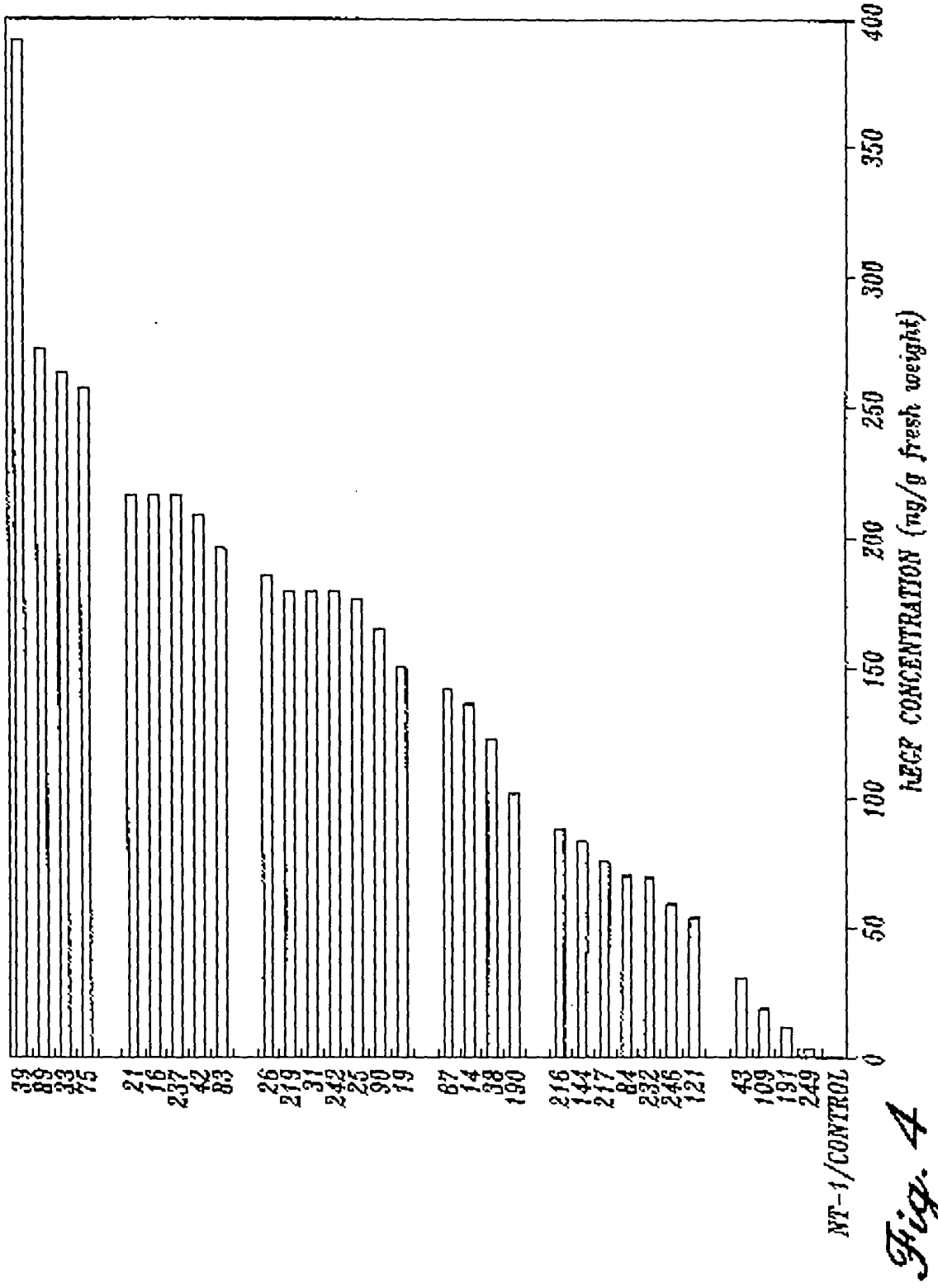

Fig. 4